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The open reading frame 5A of foxtail mosaic virus is expressed in vivo and is dispensable for systemic infection

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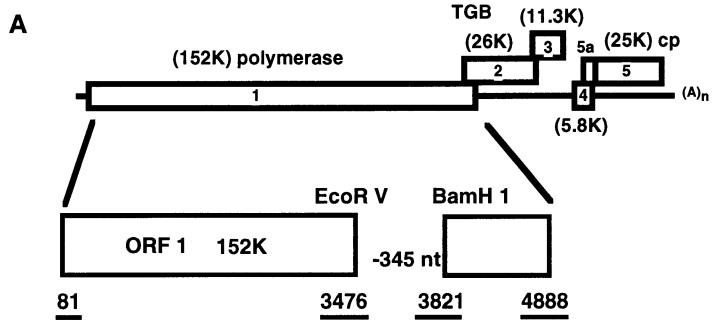
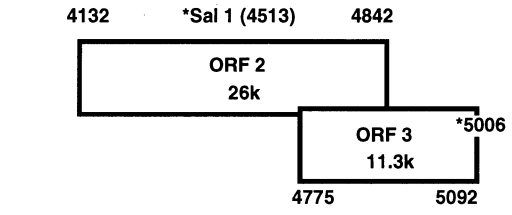
Summary. Infectious transcripts were successfully derived from full-length cDNA clones of foxtail mosaic potexvirus (FoMV). Full-length clones were constructed by RT-PCR whereby 5′ and 3′ genomic segments of 2.7 and 3.4 kb, respectively, were ligated into Bluescript II KS. The in vitro RNA transcripts were infectious to moncotyledonous (barley) and dicotyledonous (*Chenopodium amaranticolor*) plant species. Individual mutation studies on clones of each of the five major ORFs confirmed predicted gene function for the polymerase, TGB (triple gene block), and coat protein (CP) genes. Protoplast studies on expression of a unique open reading frame, ORF 5A, which initiates 143 nts upstream of the CP before it "reads through" the CP, revealed that the 5A protein was produced in vivo. Mutation analysis of the 5A ORF indicated, however, that it was not required for either replication or for productive infection of plants. However, the nucleic acid sequences encoding the extended CP segment were shown to be important for CP expression. Additional mutations in 5A had no effect on FoMV replication in protoplasts but rendered the virus noninfectious to plants. A correlation with diminished CP production from both mutant clones implies that synthesis of subgenomic CP mRNA was compromised, and this limited systemic infection.

Introduction

Foxtail mosaic virus (FoMV) is a member of the genus *Potexvirus* with a broad experimental host range. It can infect at least 56 plant species in the *Gramineae* as well as a number of species in 11 dicotyledonous families [27, 28]. The genome of FoMV consists of a capped, messenger sense ssRNA of 6,151 nucleotides

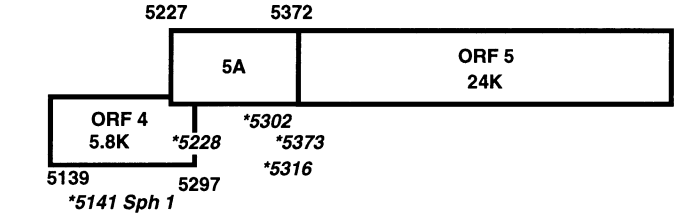


1. p152 generated by removing 345 nt between EcoR V & BamH 1 sites in ORF 1.



2. p26 : 4 nt insertion by Sal 1 restriction digest, blunt end formation, and ligation in ORF2.

3. p11.3 : 7 nt insertion (A+Mlu 1 site) at 5006 in ORF3.



4. p5.8: 4 nt deletion by Sph 1 digest, blunt ends, and ligation in ORF 4.

5. p29A : C/U substitution at 5228 in the initiation codon of 5A.

6. p29Aa: C/G substitution at 5302 in a conserved (GUUAGGG) putative cp subgenomic promoter in 5A.

7. p29Ab: 6 nt Apa 1 insertion at 5316 in 5A.

8. p24: C/G substitution at 5373 in the cp initiation codon in ORF 5.

in length and poly(A) tail [5]. The genomic arrangement of FoMV is similar to potato virus X (PVX) and other potexviruses with five major open reading frames (ORF) with presumed corresponding functions (Fig. 1). The 152 kDa protein product of the large 5′ proximal gene (ORF 1) has been partially purified and characterized [31] as an RNA-dependent RNA polymerase. The three central most genes, collectively known as the triple gene block (TGB), are presumed to mediate viral cell-to cell movement [3, 7]. The 26 kDa protein produced from the first-gene (ORF 2) of the TGB of FoMV also has been partially purified [32]. The protein binds ATP, CTP, and RNA, and has ATPase activity. The other two genes (ORF 3 and 4) in the TGB, that are predicted to produce 11.3 kDa and 5.8 kDa protein products, respectively, have not been characterized. The TGB organization is somewhat different than the prototype potexvirus, potato virus X (PVX), in that the 3′-gene (ORF 4) overlaps downstream sequences rather than overlapping with ORF 3. The significance of this difference is unknown. The coat protein gene (ORF 5; CP) is located at the 3′-terminus encoding a 24 kDa protein, although on SDS gels it appears as a *ca* 30 kDa protein [24]. It is expressed from a 0.9 kb subgenomic (sg) RNA species [5, 24].

Another significant difference between the gene organizations of FoMV and PVX is the presence of ORF 5A upstream of the CP gene. This reading frame begins at an AUG codon 143 nts 5′ of the CP gene start codon, and 95 nts before the beginning of the 0.9 kb sgRNA (Fig. 1). This ORF continues in-frame into the CP gene so that an amino-terminal extended form of CP might be produced during infection [5]. In fact, a larger protein with serologically related to the CP has been detected by in vitro translation of FoMV genomic RNA [24]. However, it is not known if this protein is produced in vivo. In addition to the established structural role of the coat protein, immuno-localization of the CP in plasmodesmata of plant cells, as well as genetic evidence, suggests a function in cell-to-cell movement [14, 25, 33, 41]. It is possible that these or other potential FoMV CP functions could require the ORF 5A amino-terminal extended form of CP.

An effective way to resolve some outstanding questions about gene function and expression of FoMV would be to produce biologically active full-length clones of the genome. Biologically active cDNA clones have been developed for many potexviruses, including PVX [16, 18], white clover mosaic virus (WCMV) [6], papaya mosaic virus (PMV) [41], clover yellow mosaic virus (CYMV) [15], strawberry mild edge-associated virus (SMYaV) [21], cymbidium mosaic virus (CymMV) [47], and bamboo mosaic virus [43] which have proved to be useful for a variety of studies.

In addition, the PVX clone has become a useful expression vector for foreign genes, but only in dicot hosts [4, 10, 11, 30, 34, 37]. Given that FoMV naturally infects a broad host range of cereals, including barely, wheat, rice, and maize,

Fig. 1. Genome organization of foxtail mosaic virus RNA and location of the mutants used in this study. **A** FoMV map and details of mutant p152, **B** mutations in ORFs 2, 3, **C** ORFs 4, 5A and coat protein gene

producing a mild to non-symptomatic infection [27, 38], we reasoned it would also be useful to develop such an infectious clone for potential transient gene expression in monocot hosts.

This paper reports on the generation of synthetic transcripts from full-length FoMV cDNA clones with infectivity comparable to wild-type RNA in both protoplasts and intact plants. Mutational analyses are described for each of the five major genes and the minor 5A ORF of FoMV.

Materials and methods

Virus source and culture

A partially purified preparation of FoMV was kindly provided GA Mackie (University of British Columbia). The virus was grown and maintained in barely (*Hordeum vulgare* var. Larker) for virion and total nucleic acid extractions.

Virus purification and RNA extraction

FoMV infected leaves were homogenized in cold 0.05 M sodium citrate buffer (1 g tissue/2 ml buffer) and filtered through several layers of cheesecloth. The sap extract was clarified with 0.5% Triton-X, followed by several differential centrifugation cycles, and the final pellet resuspended in TE, pH 8.0 or ddH_2O [22]. Virion concentration was estimated by spectrophotometry. RNA was obtained by adjusting the virion solution to 2 mg/ml in STE (0.1 M NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5), 0.5% SDS, and 100 μg/ml proteinase K, and incubating for 60 min. at 37 °C. RNA was resuspended in ddH_2O after phenol/chloroform extraction and ethanol/sodium acetate precipitation and stored at 80 °C [36].

Construction of FoMV full-length clones

FoMV full-length clones were derived by joining two PCR derived products from FoMV cDNA. The nucleotide positions are based on the published complete genomic sequence of FoMV [5]. Primers used to amplify the 5′- and 3′-FoMV genomic segments separately included two sets of primers. A 2.7 kb 5′-half segment was generated from a reverse primer that matched nts 2,990–3,010 (5-′GGAGCACCATCGCCCGCTTG-3′) and a 5′-terminus primer (5′-GCCTGCATGGGCCCAGATTTAGGTGACACTATAGAAAACTCTTCCGAAACCG-AAAC-3′) corresponding to nts 1–23 with added sequences to provide an Apa1 site and SP6 promoter; the 3.4 kb 3′-half was generated from a forward primer that matched nts 2,690–2,710 (5′-GAGGGAGCCCGCTCCTCCCA-3′) and a reverse primer that included the last ten nt of FoMV plus 29 T residues and an unique XbaI site (5′-GTGATCTAGAT_{29}ATAAGCGAT-GTGTGCATTCACC-3′). Later, a similar primer containing 80 T residues was used.

First strand cDNA was made using an anchored oligo dT primer (5′-AGCTGGATCCT_{14}-3′) and AMV reverse transcriptase (Boehringer Mannheim). PCR amplification was done using Taq DNA polymerase (Boehringer Mannheim) and the appropriate primers (10 pmol each). Thirty cycles of 95 °C (1 min), 60 °C (2 min), and 72 °C (4 min) were done to generate 5′- and 3′-genomic segments of 2.7 and 3.4 kb, respectively. PCR products were gel purified using an extraction kit (Quiagen Inc.).

The FoMV 5′-segment was ligated into a Bluescript 11 KS phagemid (Stratagene) at ApaI and EcoRI sites, and in turn, the 3′ half was then joined between the FoMV BgIII site (nt 2,788) and a phagemid XbaI site and used to transform Epicurian Coli XL2 – Blue ultracompetent cells (Stratagene). Plasmids were sequenced using Bluescript forward and reverse sequencing primers to confirm proper vector-cDNA junction sequences.

RNA transcription

All RNA transcripts were generated from XbaI linearized cDNA clones using a SP6 RNA polymerase mMessage mMachine kit (Ambion, Inc.) and 4 mM m7G(5′)ppp(5′)G cap analog as recommended by the manufacture. Transcripts for transfection of protoplasts were additionally treated with DNase. The concentration and sizes of transcripts were estimated following agarose gel electrophoresis.

Plant and protoplast inoculations

Hordeum vulgare var Larker (one-leaf stage) or *Chenopodium amaranticolor* (five-eight leaf stage) were inoculated with 5–20 μg RNA transcripts in buffer (0.5% celite, 0.5% bentonite, 0.5% sodium pyrophosphate, pH 9), and rinsed with ddH20. The plants were then kept in a plant growth chamber with 16 h. light (30 °C) and 8 h dark (25 °C) cycles and observed for at least four weeks.

Protoplast preparations from cucumber (*Cucumis sativus*) leaves were based on a protocol by Loesch-Freis and Hall [23]. Protoplasts were released from adaxial-peeled young leaves by gentle agitation in buffer [10% mannitol and 1×Aoki salts (0.2 mM KH_2PO_4, 1.0 mM KNO_3, 1.0 mM $MgSO_4$, 10 mM $CaCl_2$, 0.16 mg/ml KI, and 0.025 mg/l $CuSO_4$)] with 1.6% celulysin (Calbiochem), 0.12% macerozyme (Calboichem), and 0.1% BSA (Sigma). Protoplasts were filtered through miracloth (Calbiochem) and washed in buffer twice by centrifugation over a 20% (w/v) sucrose cushion. About 1×10^6 protoplasts were transfected with 5–10 μg of RNA transcripts by either PEG/$CaCl_2$ [23] or electroporation [26]. Transfected protoplasts were then incubated at 23 to 24 °C under constant fluorescent light between 24 and 48 h.

Virus, protein and RNA analysis

Protoplasts and leaf tissue were examined by electron microscopy of crude extracts or by serologically specific electron microscopy (SSEM) according to Derrick. [12], using a polyclonal antiserum to FoMV provided by DL Seifers, Kansas State University. FoMV proteins were detected by SDS-PAGE and Western blot analyses. Infected plant protoplasts or tissues was ground in ddH_2O (0.5 g tissue/ml) and boiled in an equal to one-half volume of Laemmli loading buffer [20]. Proteins were separated on 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and detected with the same polyclonal antibody.

Crude nucleic acid extracts were obtained from FoMV infected barely leaves ground in 0.1 M glycine, 0.1 M NaCl, and 10 mM EDTA, pH 9 and an equal volume of phenol [29]. After centrifugation, nucleic acids were precipitated from the aqueous phase with sodium acetate and isopropanol. Pellets were washed with 70% ethanol, resuspended in ddH_2O or TE and stored at 80 °C. RNA samples were electrophoreased on 1% agarose gels in 0.5×TBE (45 mM Tris-borate, 1 mM EDTA) and ethidium bromide-stained gels were electrophoretically blotted to Zeta-ProbeGT membranes as described by Bio-Rad. The membrane was UV crosslinked before overnight hybridization with a ^{32}P-labeled pFoMV probe.

Construction of mutants

Modified FoMV clones were derived from wild type cDNA clone (pF21.10) or from a subcloned FoMV segment by site-directed mutagenesis. Mutations were made by introduction, deletion or exchange of nucleotides in each gene to render them nonfunctional. Site-directed mutagenesis procedures were accomplished by subcloning a FoMV segment (nt 4,980 at KpnI to the XbaI 3′-terminus) or from BamHI (nt 3,821) to SphI (nt 5,141) sites) from the

wild type clone into pGEM3Z (Promega) and using a Transformer Site-Directed Mutagenesis kit (Clontech Laboratories, Inc.) as directed. Mutations were checked for appropriate restriction endonuclease cleavage patterns by agarose gel electrophoresis. Mutated segments were then inserted back into the appropriate restriction sites of the wild type FoMV cDNA clone.

Each gene was mutated as follows: ORF 1 (p152), a 345 nt deletion between nt 3,821 (BamHI) and nt 3,476 (EcoRV) restriction cleavage site digests; ORF 2 (p26), a 4 nt addition created by SaII (nt 4,513) cleavage, followed by filling and religation; ORF 3 (p11.3), a 7 nt insertion (AACGCGT) at nt 5,006; ORF 4 (p5.8), cleavage by SphI at nt 5141 followed by 4 nt removal and religation; ORF 5 (p24), C/G substitution at nt 5,373 in the CP gene initiation codon. Three additional modifications made to assess the function of ORF 5A (nts 5,227–5,370) included: mutant p29A, a C/U substitution at nt 5,228 in the initiation codon, mutant p29Aa, a C/G substitution at 5,302 in a conserved motif (GUUAGGG) in the putative CP subgenomic promoter, and mutant p29Ab, a six nt ApaI site (5′-GGGCCC-3′) in-frame insertion at nt 5,316.

Results

Full-length clones

The entire genomic cDNA of FoMV was successfully cloned into pBluescript II KS with the SP6 promoter and additional unique ApaI and XbaI endonuclease restriction sites at the 5′- and 3′-ends, respectively. Although entire clones were not sequenced, we did note sequence differences from the published genome [5] at several locations among several clones, both by direct sequencing and by restriction nuclease analysis. These included a C to A transversion at nt 113, an A to G transition at nt 170, a T to C transition at nt 5,748. In addition, our clone did not have a SpeI cleavage site at nucleotide 4,600 but it did have an additional NheI cleavage site at nt 5,683 and an additional 9 nt that included tandem NcoI sites at nt 5,577. The additional 9 nt and NcoI site was probably overlooked in the original sequence because NcoI was used to subclone cDNA fragments prior to sequencing [5]. Bancroft et al. [5] also reported sequence polymorphisms among different cDNA clones.

Infectious transcripts from in vitro transcribed FoMV cDNA

RNA transcripts were generated from 19 different clones, estimated to be full-length by gel electrophoresis, and inoculated to *Chenopodium amaranticolor* leaves. At five days post-inoculation (dpi), only the viral RNA positive control produced local lesions on the leaves and plant sap contained virus particles visualized by SSEM. At 21 dpi, leaves inoculated with three of the 19 clones produced several local lesions and virions were detected in leaf extracts by SSEM. When barely and *C. amaranticolor* plants were inoculated with sap from these local lesions, the passage resulted in wild type mosaic symptoms and multiple local lesions, respectively, between 4 to 7 dpi. The delayed appearance of lesions on leaves inoculated with the original transcripts suggested that some of the clones had in vivo repairable defects. The length of 3-terminal A residues of our clones ranged from 24 to 28, and previous work has documented that increasing the

Table 1. Infectivity of foxtail mosaic virus transcripts after 3′-polyadenylation[a]

Clone	*C. amaranticolor* No. local lesions	Days postinoculation
pFoMV-9	1	12
pFoMV-10	4	12
pFoMV-11	2	5
pFoMV-21	multiple	7
pFoMV-21[b]	2	6
pFoMV-26	vein clearing	21
Virion RNA	multiple	4

[a] Full-length FoMV in vitro transcripts from selected clones were polyadenylated to generate longer poly (A) tails. Plants were inoculated with 10–20 μg of the transcripts and number of local lesions recorded at days postinoculated

[b] Result from a duplicate 3′-polyadenylation experiment

length of the poly (A) tail could increase infectivity of viral cDNA transcripts [13, 15, 16, 35, 45]. This possibility was tested by selecting the three of the original 19 plasmids that gave delayed infectivity, plus two others that appeared to be noninfectious, for polyadenylation studies. Additional adenosine residues were added onto the 3′-ends of transcripts using yeast poly (A) polymerase (United States Biochemical) prior to inoculation. This treatment improved infectivity of the clones in two ways. All five clones proved to be infectious and local lesions appeared more quickly than without prior poly (A) polymerase treatment (Table 1). Even so, infectivity varied widely from experiment to experiment. To potentially overcome this variability, we sought to introduce additional 3′-A residues into one infectious plasmid clone. A new reverse primer with 80 T residues was produced and used in PCR as previously described, except that new 3′-termini were ligated into pFoMV-21 so as to replace all sequences downstream of the EcoRI site at nt 3,476 and.

Twenty full-length clones larger than the original pFoMV-21 were selected for infectivity studies. Capped transcripts were inoculated (10 μg per half leaf) on *C. amaranticolor* or on 4 to 7 barely seedlings. Control inoculations consisted of viral RNA at 0.5 μg per half leaf. Each of the new FoMV clones were infectious to plants (Table 2) with the number of local lesions approaching that of wild type RNA at 5 dpi. The proportion of systemic infections in barely at 7 dpi was more variable. In all cases, the symptoms were typical of those appearing only one to two days earlier on control plants inoculated with wild type RNA. SDS-PAGE analyses of infected leave extracts revealed a prominent protein of about 31 kDa typical of FoMV coat protein, both by Coomassie blue staining and Western blot analysis with FoMV antiserum (data not shown). Transcripts that were generated without 5′-capped (m^7GpppG) termini from selected clones were not infectious. FoMV clone pF21.10 was chosen for use in the following mutation studies to assay functional requirements of each of the five FoMV genes.

Table 2. Infectivity of foxtail mosaic virus synthetic transcripts inoculated to local lesion and systemic plant hosts[a]

FoMV clone	*C. amaranticolor* No. local lesions	Barley ~10 μg No. plants mosaic/total plants
pF21.1	100	4/4
pF21.2	56	1/5
pF21.3	86	3/5
pF21.4	52	4/5
pF21.5	20	0/5
pF21.6	15	2/4
pF21.7	20	3/5
pF21.8	55	3/5
pF21.9	50	3/5
pF21.10	100	3/4
pF21.11	130	3/4
pF21.12	160	0/5
pF21.13	50	0/6
pF21.14	40	1/6
pF21.15	50	1/6
pF21.16	80	1/5
pF21.17	30	1/5
pF21.18	25	0/5
pF21.19	30	0/7
pF21.20	12	1/7
Virion RNA (0.5 μg)	80	3/4
Mock	0	0/4

[a]FoMV in vitro transcripts were generated from selected clones and about 10 μg were inoculated to *Chenopodium* and barley leaves. Symptoms were noted as local lesions (*Chenopodium*) or as mosaic (barley). Virion RNA was inoculated as the control

Mutagenesis studies

Mutations were made in the five major genes of FoMV to confirm predicted functions from comparison with other potexviruses (Fig. 1). This was accomplished by deletion of a large region in ORF 1 (plasmid p152 in Fig. 1A), by creating frameshift mutations in ORF 2 (plasmid p26 in Fig. 1B), ORF 3 (p11.3 in Fig. 1B) and ORF 4 (plasmid p5.8 in Fig. 1C), or by eliminating the start codon with a point substitution in ORF 5 (plasmid p24 in Fig. 1C). All of these mutations were made outside of the regions where genes overlapped (ORFs 2 and 3; ORFs 4 and 5A). In addition, the 5A segment was modified to examine the effects of several types of mutations. The start codon was modified to prevent expression of the potential amino terminal extension form of CP. Another mutation was made in an octanucleotide sequence that is conserved among potexviruses and thought to be part of the putative sg promoter of the CP gene. Lastly, a six nt insertion mutation was made in ORF 5A. Infectivity of the modified transcripts was tested

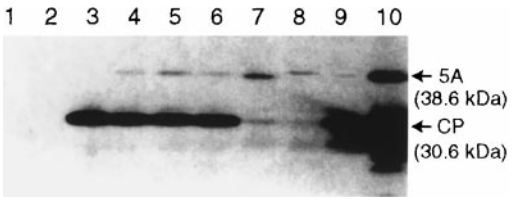


Fig. 2. Western blot analysis of foxtail mosaic virus coat protein. Total soluble protein samples were prepared from protoplasts inoculated with virion RNA and transcripts of FoMV clones. Inoculum consisted of mutants p24-ORF 5 (*2*), p29-ORF 5A (*3*), p26-ORF2 (*4*), p11.3-ORF3 (*5*), p5.8-ORF4 (*6*), p29Aa-ORF5A (*7*), and p29Ab-ORF5A (*8*). *9* is from protoplasts inoculated with infectious clone pF21.10, *10* was from protoplasts inoculated with virion RNA and *1* was mock inoculated. Arrows denote the positions of CP and ORF 5A, with the relative protein sizes, as calculated from the positions of stained protein molecular weight markers (BioRad)

on protoplasts and intact plants (Table 3). The identification of CP was confirmed by Western analysis of tissue extracts of infected protoplasts and plants (Fig. 2).

Each of the five major ORFs appeared to be necessary for whole plant infections. None of the modified clones yielded transcripts capable of producing symptoms on either barely or *C. amaranticolor* plants under conditions in which control plants produced symptoms (Table 3). Additional analyses of the same clones in protoplast infections allowed the effects of mutagenesis to be ascribed to defects in either replication, assembly or movement functions. The mutation in ORF 1 prevented accumulation of CP in protoplasts confirming its likely role in replication. The expected effects of the mutations on each of the TGB ORFs were confirmed in that protoplasts infected with modified transcripts of ORFs 2, 3, or 4 contained detectable levels of CP in each case (Fig. 2) even though they

Table 3. Modified foxtail mosaic virus transcripts inoculated to protoplasts and intact plants[a]

Gene	Clone	Protoplasts (CP production)	Barley (No. infected/total)	*C. amaranticolor* (No. local lesions)
NA	pF21.10	yes	3/3	multiple
ORF 1	p152	no	0/3	no
ORF 2	p26	yes	0/3	no
ORF 3	p11.3	yes	0/3	no
ORF 4	p5.8	yes	0/3	no
ORF 5	p24	no	0/3	no
ORF 5A	p29A	yes	3/3	multiple
ORF 5A	p29Aa	trace	no	no
ORF 5A	p29Ab	trace	no	no

[a]Each clone was mutated in the corresponding specified gene and transfected to protoplasts or inoculated to plants. The coat protein (CP) of FoMV was detected from protoplasts by western blots and the infectivity of each modified clone determined by symptoms in plants

were incapable of infecting plants. In addition, protoplasts infected with mutant p24 did not produce detectable levels of CP as expected (Fig. 2) but the mutant did replicate to near wild type levels as determined by Northern blot analysis (data not shown). We conclude from these studies that FoMV is similar to other potexviruses in that it requires both the CP gene and TGB for viral cell-to-cell movement, and that the TBG protein products are dispensable for viral replication and virion formation.

Both the 31 kDa CP and the predicted larger form of the CP with the 5A extension (38 kDa) were produced in infected protoplasts (Fig. 2). Mutations made in the ORF 5A region upstream of the CP gene also permit an initial assessment of its function. First, the mutation (p29A) designed to prevent translation of the 5A ORF had no appreciable effect on infection in plants or accumulation of CP in protoplasts (Table 3, Fig. 2, lane 3). Protoplast transfected with transcripts (p29A) containing the point mutation in the 5A start codon failed to produce this 38 kDa protein, as expected, without affecting normal CP accumulation. Further, the 5A mutant (p29Ab, Fig. 2, lane 8) that contained a two amino acid insertion (Gly-Pro) showed a distinctive alteration in migration thus confirming the origin of the 38 kDa protein from the 5A-ORF. Interestingly, reduced levels of CP relative to the 38 kDa protein were detected from protoplasts transfected with p29Aa (a point mutation within the putative CP subgenomic promoter) as well as for mutant p29Ab. This likely accounted for the inability of these mutants to infect plants because the potential to form virus particles was impaired.

Discussion

We have successfully generated infectious transcripts from full-length clones of FoMV. The infectivity (number of local lesions) of synthetic transcripts on *C. amaranticolor* leaves varied between 1/100 and 1/500 that of wild type virion RNA. Uncapped FoMV synthetic transcripts were not infectious. Potexvirus genomes have a 5′-cap structure and the use of capped transcripts has been found essential for infectivity with other viruses as well [6, 15, 16, 28, 42]. The percentage of transcripts that are actually capped during in vitro synthesis was not determined, but it is estimated to be about 67% by the Ambion kit used to generate the transcripts. In addition to reduced infectivity, symptom development was usually delayed by several days relative to virion RNA inoculations. In turn, sap from plant tissue infected by synthetic transcripts induced wild type symptoms in inoculated plants, with no time lag.

An initial assessment of FoMV gene function was made by mutagenesis and infectivity assay in plants and protoplasts and functional roles of each of the five major genes were studied by disrupting expression of particular gene products. In all cases, the outcome was similar to other potexvirus studies. We confirmed that the 5′-proximal gene is likely the viral polymerase as predicted by Rouleau et al. [31]. Evidence is also provided to confirm a role in cell-to-cell movement for ORF 2 (p26), the first gene of the TGB. Mutants in the other two genes of the TGB likewise failed to infect whole plants but replicated normally in protoplasts.

These results confirm that the TGB of FoMV is functionally analogous to the TGB of WCMV [7] and PVX [44].

FoMV CP also has an essential role in viral cell-to-cell movement as has been previously described for other potexviruses [4, 10 14, 25, 41]. The presence of a possible amino terminal extension of the CP in FoMV to form ORF 5A raised the interesting question of whether or not this altered form of CP could mediate one of these or an as yet unidentified alternate biological functions. ORF 5A was first detected by Mackie et al. [24]) as an in vitro translation product from genomic-length RNA isolated from polyribosomes. The precise genomic location of ORF 5A was identified later with the ORF reported to start at position 5227, 143 nt upstream CP gene at position 5371 [5]. In addition, the 5-terminus of ORF 5A overlaps ORF 4 by 70 nts. Thus ORF 5A could encode a CP with a 48 amino acid N-terminal extension that might be required for functions other than RNA encapsidation such as cell-to-cell or long distance virus movement.

Several counterparts to ORF 5A have been reported for other potexviruses including PMV [2, 39], CYMV [1, 40, 46], SMEaV [17], and *Plantago asiatica* mosaic virus [42] which encode 5, 45, 19, and 14 amino acids on the N-terminus of the coat protein, respectively. However, in vivo synthesis of extended CP products have not been reported for any of these potexviruses. A larger (30–31 kDa) form of CP was noted as an in vitro translation product of CYMV genomic RNA [8] but was not detected in infected protoplasts [9]. Artificially constructed CYMV transcripts representing a 29 kDa- or 28 kDa-extended coat protein gene, and the normal 23 kDa coat protein were efficiently translated in vitro, but only the smaller CP transcript was expressed in vivo when directly inoculated to plants [46]. It was suggested that the two larger polypeptides were not translated in vivo because they are not within the CP subgenomic RNA.

We now provide evidence that the 5A polypeptide is indeed synthesized in vivo in protoplast infections of FoMV. Nevertheless, the point substitution abolishing expression of ORF 5A (p29A) was still capable of infecting both protoplasts and whole plants. This implies that 5A is not essential for virus replication or systemic spread. In contrast, the clone with a point mutation (p29Aa) in the first base of the conserved octanucleotide region suspected to contain the CP subgenomic promoter was unable to infect plants. This mutant replicated in protoplasts, with markedly reduced CP accumulation but little effect on the accumulation of the 5A product. An analogous mutation in PVX also reduced CP production [19] leading to speculation that the spacing between the conserved octanucleotide and the start of the subgenomic CP was critical. We tested this possibility with FoMV mutant p29Ab which contains a six nt insertion at nt 5316, midway between the octanucleotide sequence and the CP sgRNA (nt 5323). The migration of the 5A protein from p29Ab was decreased slightly but reproducibly, as predicted by the added two amino acids, and CP accumulation in protoplasts was again severely reduced. Similarly to p29Aa, p29Ab was not infectious to plants. We conclude that while the protein product of the ORF 5A region may not be required it does encompass sequence elements essential for proper expression of CP. From this study, the 5A

protein itself seems to be dispensable for FoMV replication or spread. The possibility remains, however, that it might confer a selective advantage to the virus in its normal field setting.

The question of how the 5A protein is produced in vivo also remains unanswered. These RNA species have been detected for FoMV that include the genomic RNA and two sgRNAs, a 1.9 K RNA encoding the TGB and a 0.9 K RNA encoding the CP gene. Although the 5A protein can not be translated from the 0.9 K CP subgenomic RNA, it could possibly be expressed from the 1.9 K RNA. Recently, Verchot et al. [44] provided evidence that the first gene in the TGB of PVX is translated from a monocistronic RNA species, and the other two are produced from a bicistronic RNA species with leaky ribosomal scanning controlling expression of the last gene. It is not known if a similar RNA is produced during FoMV replication. In this regard it will be interesting to examine whether mutations in the 1.9 K sg promoter will affect expression of ORF 5A.

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